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May 14, 2003

Dr. Mary S. Wolfe
Executive Secretary
National Toxicology Program
PO Box 12233
111 T.W. Alexander Drive, MD A3-01
Research Triangle Park, North Carolina 27709

Re: National Toxicology Program Report No. TR-515, Draft Toxicology and Carcinogenesis Studies of Propylene Glycol Mono-*t*-Butyl Ether (CAS No. 57018-52-7) in F344/N Rats and B6C3F1 Mice and a Toxicology Study of Propylene Glycol Mono-*t*-Butyl Ether in Male NBR Rats (Inhalation Studies)

Dear Dr. Wolfe:

Lyondell Chemical Company (Lyondell) submits the attached comments on the National Toxicology Program's (NTP's) draft Technical Report on Toxicology and Carcinogenesis Studies of Propylene Glycol Mono-*t*-Butyl Ether. The availability of this draft technical report was announced in the Federal Register on April 16, 2003 (Vol. 68, No 73, Pages 18666-18667).

Lyondell concurs with the conclusion by NTP that there was no evidence of carcinogenic activity of propylene glycol mono-*t*-butyl ether in female F344/N rats exposed to 75, 300, or 1,200 ppm vapor. Lyondell, however, disagrees with the conclusion of equivocal evidence of carcinogenic activity in male F344/N rats and has concerns with NTP's interpretation of the tumor findings in male and female B6C3F1 mice. Our major concerns follow and the attached comments provide further information and discussion of these concerns:

- Propylene glycol mono-*t*-butyl ether should not be considered genotoxic.
- The 1,200 ppm exposure concentration was excessive and, in retrospect, exceeded the maximally tolerated dose (MTD) for propylene glycol mono-*t*-butyl ether.

In addition to these written comments on the draft Technical Report, a representative of Lyondell will be providing public comments during the May 22 meeting. The name of our speaker and other company representatives that will be attending this meeting will be forwarded in an email communication today. Lyondell Company appreciates the opportunity to provide its views on this important study report and look forward to

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further discussion at the Technical Reports Review Subcommittee meeting. In the meantime, if we can provide any further assistance or information, please contact me at (713) 309-7192 or marcy.banton@lyondell.com.

Sincerely yours,

A handwritten signature in black ink that reads "Marcy Banton". The signature is written in a cursive, flowing style.

Marcy I. Banton, DVM, PhD, DABVT
Manager, Toxicology

Attachments

**U.S. NATIONAL TOXICOLOGY PROGRAM (NTP)
DRAFT TECHNICAL REPORT (TR)
ON THE
TOXICOLOGY AND CARCINOGENESIS STUDIES OF
PROPYLENE GLYCOL MONO-*T*-BUTYL ETHER
(CAS No. 57018-52-7)
IN F344/N RATS AND B6C3F1 MICE
AND A
TOXICOLOGY STUDY OF
PROPYLENE GLYCOL MONO-*T*-BUTYL ETHER
IN MALE NBR RATS
(INHALATION STUDIES)
NTP TR 515**

**COMMENTS
OF
LYONDELL CHEMICAL COMPANY**

**COMMENTS SUBMITTED: MAY 14, 2003
SCHEDULED PEER REVIEW DATE: MAY 22, 2003**

INTRODUCTION

The Lyondell Chemical Company (Lyondell) submits these comments in response to the National Toxicology Program's (NTP's) notice (NTP, 2003) indicating that the NTP Board of Scientific Counselors Technical Reports Review Subcommittee is meeting to peer review draft Technical Reports (TR) of rodent toxicology and carcinogenesis studies. The comments address draft TR 515, which contains results of propylene glycol mono-t-butyl ether (PTB) studies.

Lyondell does not concur with the conclusion that PTB is a genotoxic material. Lyondell has reviewed the genotoxicity data for PTB and concluded that, based on the weight of evidence, PTB has not unequivocally been demonstrated to be a genotoxic material. In addition, Lyondell believes that a 1,200 ppm exposure concentration as the highest exposure level in the two-year bioassay exceeded the "maximally tolerated dose" (MTD) for PTB inhalation exposures. The following findings suggest that the hepatic function of these animals was altered due to an overwhelming PTB concentration:

1. The presence of clinical signs (ataxia, shallow breathing, lethargy) in mice during the chronic exposures.
2. Increased estrus cycle length, hepatic centrilobular hypertrophy, and increased liver weights in the subchronic study.
3. Increased incidence of altered basophilic (male rat) or eosinophilic foci (male and female mice).

These comments contain further discussion of the results of the PTB studies. To help understand some of the results, Lyondell commissioned an expert genetic toxicologist and biostatisticians to review the results and provide expert opinions. The genetic toxicity data was reviewed by Douglas B. McGregor, PhD., FIBiol., FRCPATH., Toxicity Evaluation Consultant, Lyon, France. The statistical analyses of the rat and mice tumor data were reviewed by Ciriaco Valdez-Flores, Ph.D., Larry R. Holden, Ph.D., and Robert L. Sielken, Jr., Ph.D., Sielken & Associates Consulting, Inc., Bryan, Texas. The expert remarks are included in the comments and their full reports are included as attachments to the comments.

SUMMARY

A summary of Lyondell's major concerns on the draft TR 515 report follow:

Propylene glycol mono-t-butyl ether has not been unequivocally shown to be genotoxic. Dr. Douglas McGregor has reviewed the mutagenicity studies conducted with PTB (report appended). The weight of evidence from bacterial and *in vitro* mammalian cell mutation assays, *in vitro* mammalian chromosomal aberration assays, and *in vivo* micronucleus assays suggest that PTB should not be considered a genotoxic chemical.

The 1,200 ppm exposure concentration has, in retrospect, exceeded the MTD for PTB. The 1,200 ppm exposure concentration for PTB clearly has exceeded the metabolic

and excretory capacity of the animals, resulting in altered liver function. Any increases in liver neoplasms at the 1,200 ppm exposure level are probably due to altered homeostasis. The evidence for the 1,200 ppm exposure concentration exceeding the MTD includes the following:

1. Clinical signs of ataxia, shallow breathing, and lethargy observed in the mice during the first six months of the 2-year study (but inexplicably are not present at the same exposure concentration in the two-week and 3-month studies).
2. Increased estrus cycle length in the female mice in the 3-month study.
3. Increased liver weight and hepatic centrilobular hypertrophy reported in mice and rats in the 3-month studies (also noted at 600 ppm in these studies).

The increase in estrus cycle length and liver effects suggest an alteration in circulating steroid hormone levels at the 1,200 ppm exposure concentration. Given the known effects of endogenous hormone levels on spontaneous hepatic tumor pathology (altered foci, adenomas, carcinomas), the 1,200 ppm exposure concentration, in retrospect, exceeded the MTD for PTB.

COMMENTS

1. PTB is not a mutagen or clastogen in bacterial or mammalian cells.

Dr. Douglas McGregor has reviewed all of the seven mutagenicity/clastogenicity studies available for PTB as well as the available studies for the predicted metabolites, tert-butanol and propylene glycol. His comments are as follows:

The only evidence for genetic toxicity of PTB in bacteria comes from the *S. typhimurium* TA97 experiments conducted by the NTP (2003), in which the activity is restricted to conditions under which supplementary enzymes from liver of rats or Syrian hamsters were not used. Unfortunately, unlike the other bacterial test strains used in the study, there has been no independent confirmation of the result with strain TA97. This strain is an indicator of certain types of frameshift mutation in the histidine gene and it appears that the mutation is induced by PTB itself or a contaminant, rather than by a primary metabolite of PTB, such as (as) propylene glycol or *tertiary*-butyl alcohol, or a secondary metabolite of either of these two products.

No activity of PTB or its potential primary metabolites, TBA and propylene glycol, was observed in experiments with mammalian cells *in vitro*. At most, there is weak evidence to suggest that PTB might induce micronuclei in mice of one sex, but the evidence is by no means convincing. The NTP (2003) draft report is suitably cautious in its interpretation of the micronucleus test result and a reading of it does not lead this reviewer to conclude that there is any suggestion that the *in vivo* test result is indicative of a genotoxic mode of action. The draft report quotes a review by Witt et al. (2000) in which it was demonstrated that chemicals that were clearly positive in micronucleus tests conducted to the design

that was used in NTP (2003) were highly likely to be carcinogens in rodents. Two important statements can be made regarding this conclusion of Witt et al. (2000). Firstly, it is a correlation of one type of activity with another, not a demonstration of a mode or mechanism of action. Secondly, Witt et al. (2000) determined a clearly positive result in micronucleus tests conducted to this design on the basis of the magnitude of response, or particularly when the response is observed in both sexes of mice. In the case of PTB, a statistically significant linear trend of mean MNE frequency across exposure groups was produced only in one sex, so only one of the two possible criteria for a clearly positive response was satisfied (Witt et al., 2000).

Dr. McGregor concluded,

PTB induces an increase in mutant numbers in *S. typhimurium* TA97 in the absence of liver enzymes in the incubation mixture; it has no mutagenic effect detectable in this same strain in the presence of liver enzymes or in any other bacterial strain that has been tested in either metabolic activation condition. It has not been unequivocally shown to induce a genotoxic effect *in vivo*, in mouse bone marrow cells. Furthermore, TBA and propylene glycol have not shown any genotoxic potential in a range of assays with bacteria and mammalian cells, either *in vitro* or *in vivo*.

According to Mortelmans and Zeiger (2000), a compound is considered a weak mutagen if it produces a reproducible, dose-related increase in the number of revertant colonies in one or more strains but the number of revertants is not double the background number of colonies. In the case of PTB, only the 10,000 micrograms/plate (3,774 micrograms/ml) is above double the background rate of revertants (the background rate is 135-149, thus twice the background rate is 270-298 revertants). The revertant counts in each of the duplicate trials at 10,000 micrograms/plate is just above twice background, with 325 revertants counted in the first run and 318 counted in the second run.

2. Exposure of rats and mice to 1,200 ppm PTB exceeded the requirements for the MTD.

The exposure of mice to 0, 75, 150, 300, 600, or 1,200 ppm PTB in the two-week studies states (Page 73) that "There were no clinical findings related to PTB exposure". In the report for the 3-month study, clinical signs related to inhalation exposure to PTB are not mentioned in the results. However, under "Exposure Concentration Selection Rationale" (Page 77), it states "There were no treatment-related mortality, reductions in body weight gain, or clinical findings in B6C3F1 mice exposed to 0, 75, 150, 300, 600, or 1,200 ppm PTB."

When this same strain of mouse was exposed to 1,200 ppm in the 2-year study, clinical findings included ataxia, shallow breathing, and lethargy during the first six months of the study (Page 81). Apart from the obvious animal welfare concerns, how can these clinical findings (most likely related to acute exposure to 1,200 ppm PTB) be

found during the 2-year study but not in the two-week and 3-month studies with similar exposures to 1,200 ppm PTB? Clearly these acute clinical signs must have been present during the exposures for the two-week and 3-month studies. The fact that they were not recorded (or reported) brings into question not only the quality of the studies but also the basis for the exposure selection rationale for the 2-year study.

The exposure selection rationale appears to be based on mortality, body weight gain, clinical findings (see above), increased liver weights and hepatic centrilobular hypertrophy at 600 and 1,200 ppm. The hepatic effects at 1,200 ppm were not considered sufficiently severe to preclude use of that exposure as the highest concentration. No mention is made of clinical signs since none were reported for the 3-month study. The rationale for the exposure selection for the mouse 2-year study (page 77) also mentions the use of the toxicokinetics data reported in Appendix N. The toxicokinetic data only included exposures to 75, 300, and 1,200 ppm. The 1,200 ppm blood data clearly show that this exposure concentration is overwhelmed the metabolic and excretory capacity of the mice with blood levels 12 minutes following exposure (C_0) of 547 $\mu\text{g/g}$ in the male mice and 800 $\mu\text{g/g}$ in the female mice. It is interesting to note that if this exposure would have been to ethanol, the female mice would have been considered legally drunk (0.08 mg %) while the male mice would have been simply “under the influence” (above 0.05 mg%).

The report states that these exposure concentrations were selected for the 2-year study since one was most likely in the linear range (75 ppm), one was near the linear portion (300 ppm) and one was well above the linear range (1,200 ppm). In reality, since these were the only exposure levels tested in the toxicokinetic study, these were the only concentrations available for selection. However, the clear increase in blood levels between 300 and 1,200 ppm (a 4-fold increase in vapor concentration with a 36-52 fold increase in blood concentration) should have indicated that this exposure concentration exceeded the metabolic and excretory capacity of the animals.

The observed clinical signs, estrus cycle, and hepatic effects and the toxicokinetic data clearly indicate that 1,200 ppm exceeded the MTD for PTB in the 2-year study.

The exposure concentration selection, however, could not have been conducted any differently given the available data. The clinical signs associated with exposure to 1,200 ppm had not been reported in the two-week and 3-month studies, even though they surely were present. The toxicokinetic data only included these three exposure concentrations; no other data was available at other levels. The presence of liver effects at 600 ppm in the 3-month study was the only indication that altered liver function was present in animals at the lower exposure concentrations.

Another indicator of altered liver function in female mice exposed to 1,200 ppm PTB was longer estrus cycles in exposed mice compared to chamber controls animals (Table J4). Specifically, the female mice exposed to 1,200 ppm had longer periods of diestrus and estrus and shorter periods of proestrus and metestrus. These changes are dependent on circulating levels of endogenous estrogens and progesterone, two steroid

hormones known to be metabolized and cleared by the liver. As the metabolic capacity of the liver is induced, these hormones are cleared more rapidly from the circulation and subsequently require longer periods of time to attain physiological concentrations necessary to change the stage of the estrus cycle. Since maintaining normal metabolic clearance rates for endogenous steroid hormones is a function of the liver, altered hepatic metabolism and clearance (both increases and decreases) indicate an adverse effect on liver function. The consequences of altered circulating endogenous steroid levels on hepatic function as well as affecting the spontaneous incidence of altered hepatic foci, adenomas, and carcinomas has been the subject of intense investigations for the past decade. Given what is known regarding circulating endogenous hormone levels on spontaneous liver tumor incidence in mice and rats, exposure concentrations should not be selected that affect parameters dependent on these same hormone levels.

The MTD has traditionally been based on simple parameters of body weight gain and survival. However, our current knowledge of homeostasis provides us with more sensitive measurable parameters. In the 40-plus years of recent research into the science of toxicology, it has become apparent that toxicity can also become manifest on the basis of altered organ function (in this case the liver with subsequent effects on endpoints dependent on circulating hormone levels), which can occur without effects on body weight gain or survival. These alterations in homeostasis, coupled with the toxicokinetic data generated prior to exposure level selection, should have been used to select the highest exposure level for PTB in the 2-year study. Since the 600 ppm exposure concentration did not affect estrus cycle length yet still resulted in increased liver weights and centrilobular hypertrophy, this exposure concentration may have been more appropriate as the highest exposure concentration for the 2-year study.

EXPOSURE SELECTION IN RATS

The two-week and 3-month studies in rats focused primarily on the kidney effects of PTB (for good reason). However, the liver weights were increased in both the male and female rats exposed to 600 and 1,200 ppm for three months. Histopathological changes to the liver from the 3-month studies have not been included in the report (see pages 54-58); only lesions in the kidney and nose have been included. Therefore, it is not possible to determine what changes to the liver were present at the end of the 3-month study or if they were even considered when selecting the exposure levels for the 2-year study.

The exposure concentration selection rationale (page 58) mentions that the liver was considered a target organ based on increases in liver weights at 600 and 1,200 ppm in the male and female rats. However, the only histological changes that were considered were those related to kidney toxicity. The use of toxicokinetic data in the exposure selection was limited to the three concentrations tested (see above discussion of mice data) and again indicated a 16-18 fold increase in blood concentration in response to a 4-fold increase in exposure concentration from 300 to 1,200 ppm (Appendix N).

The focus on kidney toxicity is understandable, given the possibility of these effects affecting the survival of the rats (particularly male rats) over the 2-year test period. However, in retrospect, it is apparent that the 1,200 ppm exposures exceeded the metabolic and excretory capacity of the liver (based on the toxicokinetic data) and probably resulted in altered liver function in the male and female rats in the 2-year study.

WHY IS THE ISSUE OF ALTERED LIVER FUNCTION IMPORTANT TO THE INTERPRETATION OF THE 2-YEAR STUDY RESULTS?

The issue of a possible effect of altered circulating levels of steroid hormones due to induced metabolism following PTB exposure on the incidence of altered liver foci, adenomas, and carcinomas in mice and rats is very complex. In addition, it is also well accepted within the scientific community that mice and rats respond differently to these sex-specific differences (many excellent reviews have been published, which will not be repeated here). This may explain the differing response in the male and female mice when compared to the male and female rats. What is clear is that the 1,200 ppm exposure group overwhelmed the metabolic capacity of the liver and altered endpoints dependent upon normal circulating levels of steroid hormones, thus exceeding the MTD.

The statistical analysis conducted by Sielken & Associates Consulting, Inc. (see below and appended) indicate that without the data from the 1,200 ppm groups, PTB does not increase the incidence of liver neoplasms in mice and rats:

The conclusions drawn by the NTP are based solely on some adverse liver effects observed in male and female mice exposed to 1,200 ppm. The mice exposed to 1,200 ppm showed signs of toxic effects and the NTP should consider excluding them when analyzing the incidences of liver neoplasms. If this highest dose group were dropped from the statistical analyses, the only conclusion that could be drawn from the NTP report is that there is no evidence of carcinogenic activity in male or female mice. NTP's conclusion also ignores some well-known pitfalls of statistical analysis that could have led to spurious statistically significant findings.

The conclusion of equivocal evidence drawn by the NTP is based primarily on the purported increasing slope on hepatocellular adenomas in male rats. However, none of the incidences in the exposed rats are significantly different from the incidence observed in the chamber control male rats. In fact, the incidences at the two lowest dose groups are smaller than the incidence observed in the chamber control rats. The apparently significant trend is more likely a result of random differences in the observed incidences and the unequal spacing between doses. The "significant" trend disappears if the unequal spacing between doses is taken into account by replacing them by ranks. The trend also disappears if the male rats in either the lowest or the highest dose group are excluded from the analysis. These facts make it unlikely that

the trend is due to a real chemical effect. Rather, it is more likely due to the peculiar characteristics of the data.

Since exposure to 1,200 ppm PTB clearly exceeded the metabolic and excretory capacity of the animals and resulted in extremely high blood concentrations leading to altered hepatic function, it should be considered as having exceeded the MTD. Without the data from the 1,200 ppm PTB, there is no credible increase in liver neoplasms in either the rats or mice.

3. **Toxicokinetic data for mice exposed to 1,200 ppm PTB are non-linear and result in disproportionately high blood concentrations. Aberrant tissue responses, including the development of hepatic tumors arising as a consequence of chronic toxicological insult, would not be unexpected under such conditions.**

Selection of the upper exposure concentration for the main (bioassay) segment of these investigations was based upon the occurrence of treatment-related toxicological and histopathological lesions in rats and mice following sub-chronic exposure to PTB. As a result, the kidney and liver were identified as potential target organs in the rat while changes in the mouse were restricted to the liver only. However, since none of these responses appeared particularly severe in either species, it was concluded that 1,200 ppm PTB (the highest exposure concentration used in the 3-month study) would be acceptable as the highest concentration in the 2-year study.

Lower concentrations were selected using toxicokinetic data obtained from rats and mice following a single 6-hour exposure to 75, 300 or 1,200 ppm PTB. Based upon this information, it was concluded that systemic elimination (blood clearance) of PTB at 1,200 ppm, and probably at 300 ppm also, was outside the linear range for both species. In the rat, 75 ppm was considered most likely within the linear range, while it was unclear whether this concentration was inside or outside the linear range in the mouse.

Toxicokinetic data presented in Appendix N (*Single-Exposure Toxicokinetic Studies in F344/N rats and B6C3F1 Mice*) of the draft report demonstrate this non-linear response.

The exposure levels used in these investigations (75, 300 and 1,200 ppm) increased in a numerical ratio of 1: 4: 16. In contrast, the concentration of PTB in blood from exposed rats increased in the ratio of 1: 6: 115 in male rats or 1: 6: 98 in females, while blood levels in mice were in the ratio 1: 9: 323 for males and 1: 11: 567 for females. These data demonstrate, quite clearly, that a 16-fold increase in external exposure concentration resulted in a 100 to 550-fold increase in internal dose. In absolute terms, mean blood concentrations in high dose mice (547 or 800 µg/g blood for males or females, respectively) were 1.7 to 2.1-fold greater than those present in high dose rats (311 or 386 µg/g blood, respectively).

Estimated Michaelis-Menten kinetic (clearance) constants following a single 6-hour exposure to 1,200 ppm PTB gave a K_m of approx. 200-270 $\mu\text{g/g}$ and V_{\max} of 2.4-2.8 $\mu\text{g/g}$ per minute in the rat, while equivalent values in the mouse were 78-120 $\mu\text{g/g}$ (K_m) and 4-5 $\mu\text{g/g}$ per minute (V_{\max}). While these observations suggest possibly faster clearance of PTB by the mouse (lower K_m , greater V_{\max}) it is unclear if this would have had a significant impact on systemic dose given the very high blood concentrations that were recorded.

Taken together, these considerations demonstrate clear differences in circulating (systemic) concentrations between rats and mice following a single 6-hour exposure to 1,200 ppm PTB which, if sustained over the 2-years of a cancer bioassay, would have resulted in a significantly greater total received dose in the high dose mice compared to rats. The toxicological consequences of effectively 'saturating' an animal with PTB appears to have been overlooked by NTP during the dose-setting stage of this study: aberrant tissue responses would not be unexpected under such conditions, including the development of tumors arising as a consequence of chronic hepatic insult. These points suggest that the results from this study provide no more than equivocal evidence of carcinogenicity and should be highlighted in the report.

4. **Changes in renal histopathology observed in rats from these studies are fully consistent with accumulation of $\alpha_2\mu$ -globulin and exacerbation of Chronic Progressive Nephropathy, two rodent-specific lesions that have no human counterpart.**

When discussing renal findings for rats from the 2-year study, the report notes (page 68, paragraph 2) that "*age-related chronic nephropathy occurred in all exposed males and in most exposed females...*" and that "*...the severities of nephropathy increased with increasing exposure concentration in males and females, and were significantly increased in all exposed groups of males and in 1,200 ppm females.*" It is also noted that "*nephrotoxic chemicals frequently exacerbate the severity of nephropathy in both sexes of F344/N rats*". Overall, this information clearly indicates that long-term inhalation exposure to PTB exacerbated development of Chronic Progressive Nephropathy (CPN), a spontaneous age-related disease of laboratory rats that has no strict counterpart in humans.

It is also noted in the discussion (page 97, paragraph 2) that "*propylene glycol mono-*t*-butyl ether meets the three criteria listed by the USEPA to link the $\alpha_2\mu$ -globulin process and the observed renal neoplasm outcome*", including increased number and size of hyaline droplets in proximal tubules in treated males, presence of $\alpha_2\mu$ -globulin in the droplets and the presence of granular casts, mineralization and hyperplasia. These observations are consistent with the demonstrated increase in renal $\alpha_2\mu$ -globulin concentration observed in the 3-month study (Table 6) together with the known metabolic conversion of PTB to *tert*-butanol, which has independently been shown to increase the presence of $\alpha_2\mu$ -globulin-immunoreactive material in male rat kidney (Borghoff *et al.*, 2001).

It is therefore surprising that NTP should cast doubt on the mechanism underlying the renal tumors observed in this study by stating (page 97, paragraph 2) that these findings "...only satisfy some of the International Agency for Research on Cancer (IARC) criteria for kidney carcinogenicity..." and inferring that some genotoxic event was responsible. As noted above, the evidence supporting a conclusion of a genotoxic mechanism is extremely weak. It is also inconsistent that NTP should suggest (page 98, top of page) that "*additional research is needed to characterize the binding of propylene glycol mono-t-butyl ether to $\alpha_2\mu$ -globulin and to clarify the role of the $\alpha_2\mu$ -globulin mechanism in the observed tumor outcome in male rats in the current study*": sufficient information to support this link exist already.

For avoidance of doubt, NTP should state clearly and unambiguously in the Abstract and in the Discussion and Conclusions that these changes in renal histopathology are fully consistent with accumulation of $\alpha_2\mu$ -globulin (males) and exacerbation of CPN (both sexes), and that neither of these events has a clear human counterpart.

5. Corneal mineralization noted in high dose rats and mice from these studies appears consistent with the known irritant effects of propylene glycol mono-t-butyl ether on the eye.

As noted in the Introduction (page 22, top of page), neat liquid PTB is a severe eye irritant. It is therefore not surprising that corneal mineralization was observed in rats and mice exposed to 1,200 ppm PTB for 2-years.

6. There are established structural, metabolic and toxicological differences between propylene glycol ethers and ethylene glycol ethers. Comparison of the health hazards of PTB and these unrelated substances is inappropriate. Therefore, any reference to ethylene glycol ethers should be removed from the report.

The Introduction (pages 20 to 25) contains discussion of the mammalian toxicology of PTB and other glycol ethers, including propylene glycol monomethyl ether and ethylene glycol monomethyl, monoethyl and monobutyl ethers. PTB is a member of the family of propylene glycol ethers. Propylene glycol ethers have a secondary alcohol group and no primary alcohol groups. As such, the propylene glycol ethers are primarily metabolized by microsomal enzymes with propylene glycol and the constituent alcohol being the only metabolites. For PTB, the metabolites would be propylene glycol and t-butanol.

In contrast, the ethylene glycol ethers always contain a primary alcohol and this fact explains the metabolic pathway for this family of glycol ethers. Alcohol and aldehyde dehydrogenase act on the primary alcohol moiety with an alkoxyacetic acid metabolite being formed. Since the alkoxyacetic acid moiety is the metabolite responsible for many of the toxicity characteristics of this series of glycol ethers, the

metabolic pathways for this series of glycol ethers are of paramount importance. The report acknowledges this fact, as noted by NTP (page 23), "*Differences in toxicity among ethylene and propylene glycol ethers are largely attributed to the (sic) differences in their metabolism. While propylene glycol ethers undergo O-dealkylation to form propylene glycol, ethylene glycol ethers are metabolized to the corresponding toxic alkoxyacetic acids....*" These metabolic and toxicologic differences between the two series of glycol ethers suggests that information relating to ethylene glycol ethers adds no useful information and should be removed from the final report.

It is overly simplistic to group chemicals simply by the presence of certain terms within the words used to describe them. With the example cited above, the differing metabolic pathways (due to the presence or absence of a primary alcohol group) suggests that grouping the propylene and ethylene glycol ethers together when discussing toxicity parameters is inappropriate and ignores the last twenty years of toxicity research for these groups of chemicals. Similarly, toxicologic comparisons between PTB and propylene glycol monomethyl ether are of doubtful utility since the parent alcohols (*tert*-butanol, tertiary alcohol; methanol, primary alcohol) exhibit distinct chemical, metabolic and toxicological characteristics.

SPECIFIC COMMENTS

Metabolism of PTB yields both propylene glycol and *tert* butanol

The Introduction section notes (page 19, paragraph 2): "*In addition to being conjugated, propylene glycol mono-t-butyl ether is also hypothesized to be partly metabolized by O-dealkylation to propylene glycol...*" For completeness, the report should also note that *tert*-butanol is also formed and further metabolized to 2-methyl-1,2-propanediol, 2-hydroxyisobutyrate and other minor products (Bernauer *et al.*, 1998; Amberg *et al.*, 2001)

REFERENCES

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- Borghoff, S.J., Prescott, J.S., Janszen, B.B., Wong, B.A., and Everitt, J.I. (2001). Alpha 2u-globulin nephropathy, renal cell proliferation and dosimetry of inhaled t-butyl alcohol in male and female F-344 rats. *Toxicol. Sci.* **61**(1): 176-186.
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ATTACHMENTS

- Attachment A D.B. McGregor: Expert Report: The Genetic Toxicity of Propylene Glycol Mono-tertiary-Butyl Ether and its Contribution to the Mode of Action of any Carcinogenic Activity of this Compound. May 14, 2003.
- Attachment B C. Valdez-Flores, L.R. Holden, and R.L. Sielken, Jr.: Expert Report: Review of the Statistical Analyses of the Two-Year Rodent Studies in the NTP Technical Report on the Toxicology and Carcinogenesis Studies of Propylene Glycol Mono-*t*-Butyl Ether (CAS No. 57018-52-7) in F344/N Rats and B6C3F1 Mice and a Toxicology Study of Propylene Glycol Mono-*t*-Butyl Ether in Male NBR Rats (Inhalation Studies) NTP TR 515 Publication No. 03-449. May 13, 2003.

ATTACHMENT A

**EXPERT REPORT:
THE GENETIC TOXICITY OF
PROPYLENE GLYCOL MONO-TERTIARY-BUTYL ETHER
AND ITS CONTRIBUTION TO THE MODE OF ACTION
OF ANY CARCINOGENIC ACTIVITY OF THIS COMPOUND**

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**Prepared for
Lyondell Chemical Company**

The Genetic Toxicity of Propylene Glycol Mono-*tertiary*-Butyl Ether and its Contribution to the Mode of Action of any Carcinogenic Activity of this Compound

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This document consists of nine (9) numbered pages.

The U.S. National Toxicology Program (NTP) has performed two-year inhalation exposure studies of Propylene Glycol Mono-*tertiary*-Butyl Ether (PGtBE) in F344/N rats and B6C3F₁ mice (NTP 2003). This report also described studies on the toxicity of PGtBE in male NBR rats, and a number genetic toxicity studies, as well as providing a summary of the metabolism, absorption and distribution of the substance in rats. Detailed discussion of PGtBE carcinogenicity will be dealt with in other documents. The issue to be addressed here is that, if PGtBE is carcinogenic, is the mechanism of action based on genetic toxicity?

General (Draft) Conclusions of the NTP (2003) Study

Under the conditions of the 2-year inhalation route study, exposure to PGtBE resulted in non-neoplastic lesions of the kidney in male rats, the liver and nose in male and female rats, the liver in male and female mice and the eyes in female rats and mice. There was evidence of α_2 -globulin nephropathy in the 300 and 1200 ppm groups of male rats and chronic progressive nephropathy occurred in all groups of rats, the severity of which showed a dose dependent increase in male rats and was also significantly increased in female rats of the 1200 ppm group. The draft report concludes that, based on a marginally increased incidence of renal tubule neoplasms and hepatocellular adenomas, there was *equivocal evidence of carcinogenic activity* of PGtBE in male F344/N rats, while there was *no evidence of carcinogenic activity* of PGtBE in female F344/N rats exposed to 75, 300 or 1,200 ppm. The draft report also concludes that there was *clear evidence of carcinogenic activity* of PGtBE in male and female B6C3F₁ mice based on increased incidences of liver neoplasms.

Metabolism of PGtBE

This topic is a necessary consideration for the genetic toxicity evaluation because different results have been obtained under different metabolic conditions. The data summarised below are derived, with modification, from NTP (2003). Only data deemed to be relevant to the objective of this opinion are reproduced.

Following the oral administration of radiolabelled PGtBE doses ranging from 3.8 to 377 mg/kg bw to male Fischer F344/N rats (NTP, 1994), 87% to 100% of the dose was eliminated by 72 h and less than 6% of the dose remained in the carcasses. From 48% to

67% of the dose was eliminated in the urine, consisting of 23% to 52% PGtBE glucuronide and 7% to 13% as PGtBE sulphate.

Direct metabolic studies have not been conducted with PGtBE, but a scheme has been suggested whereby PGtBE is partly conjugated to form sulphates and glucuronides that are excreted in the urine and partly dealkylated to propylene glycol via microsomal *O*-dealkylation in a manner similar to propylene glycol mono-*n*-butyl ether (PGBE) (Verschuuren,1996). A secondary alcohol such as PGtBE would not be expected to be a good substrate for alcohol dehydrogenase (Miller et al., 1983). Propylene glycol is in turn further metabolised to lactic acid and pyruvic acid, which enter the tricarboxylic acid cycle, resulting in ultimate elimination as carbon dioxide (Miller,1987). The excretion of 22% to 26% of the administered oral dose of PGtBE as expired carbon dioxide in rats was reported by NTP (1994). This finding is consistent with the metabolism of PGtBE to carbon dioxide via *O*-dealkylation, but this pathway remains to be demonstrated. Biliary excretion studies demonstrated that 40% of a radiolabelled intravenous dose was recovered in the bile as PGtBE glucuronide. However, only 11% or less of the dose was recovered from the faeces, suggesting that the metabolite was hydrolysed in the intestine and the released PGtBE reabsorbed.

From this information, it would appear that the substances of possible importance to genetic toxicity are PGtBE itself, propylene glycol and *tertiary*-butyl alcohol.

Genetic Toxicity Data

Bacteria.

In the NTP (2003) investigation, PGtBE was tested at doses up to 10,000 µg/plate. At doses of about 1,000 µg/plate and higher it was reproducibly mutagenic in *S.typhimurium* strain TA97 in the absence of liver S9 activation enzymes. No significant response was observed in strain TA97 in the presence of rat or hamster liver S9 enzymes, in strains TA98, TA100 or TA1535 with and without S9 or in strain TA1537 without S9 (it was not tested against TA1537 in the presence of the liver enzyme preparations).

These NTP (2003) results confirm those obtained and presented in other, unpublished reports with PGtBE, except that the strain TA97 was not tested in these unpublished studies (Barfknecht, 1986; Jones, 1987). The NTP results with *S.typhimurium* TA97 in the absence of liver S9 activation enzymes were as follows:

Dose (µg/plate)	Colonies/plate (Expt.1)	Colonies/plate (Expt.2)
0	135 ±3.8	149±0.3
100	123±3.8	146±8.4
333	131±8.3	163±5.0
1000	150±5.5	227±22.3
3333	248±18.3	247±10.7
10000	325±18.9	318±26.6

It is clear from the data presented in the report that the mutagenic activity observed with this strain was quenched by some unidentified mechanism in the presence of liver enzyme preparations.

One possible major metabolite of PGtBE, *tertiary*-butyl alcohol, has been tested in bacteria, but no mutagenic activity was demonstrated in *S. typhimurium* TA97a (Williams-Hill et al., 1999). In addition, these authors found no significant response with strains TA98 or TA100, but a significant response was obtained using the DNA repair proficient strain, *S. typhimurium* TA102. This result has not been reproduced, however, in two independent and well-conducted studies with the same bacterial strain (May & Watson, 2000; Callander, 2003). In these latter two studies, no effect of TBA was observed in either the presence or absence of a rat liver S9 mix preparation and, additionally, Callander (2003) showed that using deionised water as the solvent rather than DMSO had no effect on the negativity of the result at dose levels up to 5000 µg/plate. Finally, no significant response was observed in *S. typhimurium* TA98, TA100, TA1535 or TA1537 when TBA was tested at dose levels up to 10000 µg/plate in the presence and absence of rat and Syrian hamster liver S9 preparations (Zeiger et al., 1987).

Propylene glycol has been tested for mutagenic activity in bacteria at doses of up to 10000 µg/plate in *S. typhimurium* TA92, TA94, TA98, TA100, TA1535 and TA1537 (Pfeiffer & Dunkelberg, 1980; Ishidate et al., 1984). No mutagenic activity was found. The absence of data using the strain *S. typhimurium* TA97 is not thought to be important, given that the available mutagenicity data with PGtBE are suggesting that either PGtBE itself, or a contaminant, is the active substance.

Mammalian Cells in Culture

PGtBE did not induce either sister chromatid exchanges (SCE) or chromosomal aberrations in Chinese hamster ovary (CHO) cells in the presence or absence of rat liver S9, according to the NTP (2003) studies. The latter results confirm those of unpublished studies in which PGtBE did not induce chromosomal aberrations in CHO cells (Allen et al., 1987) or human peripheral lymphocyte cultures at concentrations up to 5000 µg/ml (Scheres, 1991). In addition, PGtBE did not induce mutations at the *tk*⁺/*tk* locus in mouse lymphoma L5178Y cells when tested at concentrations up to 5000 µg/ml (Verspeek-Rip, 2001).

There have been a number of studies with the potential major metabolites, TBA and propylene glycol.

TBA was tested in the mouse lymphoma L5178Y cell *tk*⁺/*tk* forward mutation assay (McGregor et al., 1988) at concentrations up to 5000 µg/ml in the presence and absence of a rat liver S9 mix preparation. The experiments were conducted twice under each activation condition. In one experiment without S9 mix, there was a small increase in mutant fraction (1.6-fold the control value) at a single dose level (5000 µg/ml). This was not reproduced in the second experiment without S9 mix and was not observed in either

of the experiments performed in the presence of S9 mix. It was concluded that *tertiary-butyl* alcohol is not a mutagen.

TBA was tested in a sister-chromatid exchange assay in CHO cells (NTP, 1995), at concentrations up to 5000 µg/ml in the presence and absence of a rat liver S9 mix preparation. The experiments were conducted twice under each activation condition. In one experiment without S9 mix, there was a small increase in sister-chromatid exchange frequency (1.2-fold the control value) at a single dose level (5000 µg/ml). This was not reproduced in the second experiment without S9 mix and was not observed in either of the experiments performed in the presence of S9 mix. It was concluded that TBA does not induce sister-chromatid exchanges in this system.

As part of the same study, TBA was tested in a chromosomal aberrations assay in CHO cells (NTP, 1995) at concentrations up to 5000 µg/ml in the presence and absence of a rat liver S9 mix preparation. The experiments were conducted twice under each activation condition. In the second experiment without S9 mix, there was an increase (judged by the investigators/NTP to be *equivocal*) in the frequency of aberrations at a single dose level (5000 µg/ml). This had not been observed in the first experiment without S9 mix and was not observed in either of the experiments performed in the presence of S9 mix. It was concluded that TBA does not induce chromosomal aberrations in this system.

In studies with propylene glycol, no increase in chromosomal aberrations was found in human lymphocyte cultures that had been treated with propylene glycol concentrations up to 50 mM in both the presence and absence of an exogenous metabolic activation system (Erdoelchemie, 1990). An increase in the frequency of chromosomal aberrations was observed in CHL cells in the absence of any exogenous metabolic activation system, but only at a cytotoxic concentration of 420 mM (Ishidate et al., 1988). It is noted that in both of these studies, the recommended upper concentration limit of 10 mM was grossly exceeded.

Mammalian Cells *In Vivo*

It is asserted in the NTP (2003) draft report that PGtBE induced a small but significant increase in the frequency of micronucleated normochromatic erythrocytes (MNE) in peripheral blood of female mice in a 3-month study, following exposure to vapour concentrations of from 75 to 1200 ppm. No significant increase in micronucleated normochromatic erythrocytes was seen in male mice, and the percentages of polychromatic erythrocytes were similar in male and female mice of the exposed and chamber control groups. These statistical assertions are not challenged, particularly if adjustments for multiple comparisons are not applied (it appears that such an adjustment is part of the ILS, 1990 analytical package used). The results for male and female mice were as follows.

Concentration (ppm)	MNE/1000 NE (Male mice)	MNE/1000 NE (Female mice)
Chamber control	1.05±0.23	0.70±0.15

75	0.95±0.17	0.95±0.20
150	1.25±0.20	0.75±0.20
300	1.00±0.17	0.60±0.18
600	0.55±0.17	1.00±0.15
1200	1.10±0.15	1.25±0.17

In male mice, no statistically significant differences were found in pair-wise comparisons of any group with the chamber controls. Also, a test for trend was not statistically significant and there was no indication of a dose-response relationship that failed to meet a statistically significant level.

In females, however, the results of pair-wise comparisons of treated groups with the chamber controls showed that there was a statistically significant difference for the 1200 ppm group ($p = 0.039$). A test for trend was also statistically significant ($p = 0.021$), but this result is driven by the effect of the 1200 ppm group, there being no statistically significant differences in pair-wise comparisons of the chamber controls (0.70 ± 0.15) with the other dose level groups up to and including 600 ppm (1.00 ± 0.15 MNE/1000 NE) ($p = 0.152$), which is almost identical to the 75 ppm group result (0.95 ± 0.20 MNE/1000 NE) ($p = 0.192$). It is also noted that the range of mean values in the different groups of males is very similar to the range observed in females, suggesting that the finding in female mice cannot be readily accepted as being biologically significant. These doubts can be removed (either way) only by results from further experiments. Consequently, while recognising the statistical analysis as it stands, it is the opinion of this reviewer that the results of this experiment provide only very weak evidence for an effect of exposure to PGtBE on the frequency of micronucleated erythrocytes.

TBA was tested for its potential to induce micronuclei in peripheral blood erythrocytes of male and female B6C3F₁ mice continuously exposed for 13 weeks to drinking water containing up to 40 mg/ml TBA (NTP, 1995). The percentage of micronucleated normochromatic erythrocytes varied in male mice from 0.09 ± 0.01 in the control group to 0.06 ± 0.03 in the 40 mg/ml group and, in female mice, from 0.06 ± 0.01 in the control group to 0.07 ± 0.01 in the 40 mg/ml group. Although a trend test applied to the results from female mice was significant ($p = 0.020$), no pair-wise comparisons indicated a statistically significant difference from the controls and it was concluded that TBA does not induce micronuclei in this *in vivo* system.

Similarly, propylene glycol did not produce an increase in chromosomal aberrations in bone marrow cells of male rats treated orally by gavage at dose levels up to 5000 mg/kg bw either once or daily for 5 consecutive days (Litton Bionetics, 1974). Also, no increase in the frequency of micronucleated polychromatic erythrocytes was observed in the bone marrow of mice treated by intraperitoneal injection with propylene glycol at dose levels up to 15000 mg/kg bw on a single occasion 18 h before marrow sampling (Hayashi et al., 1988).

Discussion

Bacteria.

The only evidence for genetic toxicity of PGtBE in bacteria comes from the *S. typhimurium* TA97 experiments conducted by the NTP (2003), in which the activity is restricted to conditions under which supplementary enzymes from liver of rats or Syrian hamsters were not used. Unfortunately, unlike the other bacterial test strains used in the study, there has been no independent confirmation of the result with strain TA97. This strain is an indicator of certain types of frameshift mutation in the histidine gene and it appears that the mutation is induced by PGtBE itself or a contaminant, rather than by a primary metabolite of PGtBE, such propylene glycol or *tertiary*-butyl alcohol, or a secondary metabolite of either of these two products.

The SIDS dossier 1-Methoxypropan-2-ol (CAS No. 107-98-2) DATE: October 12, 2000, Sponsor Country: U.S.A. (i.e., propylene glycol mono-methyl ether, PGME) records that no bacterial mutagenicity was observed with PGME in experiments reported from three independent studies with *S. typhimurium* strains TA1535, TA1537, TA1538, TA98 and TA100 at dose levels up to 5000 µg/plate in two of them and 6250 µg/plate in the third. Clearly, these data do no more than support the lack of effect in the same strains with PGtBE, while adding nothing to clarify the positive response found by the NTP using strain TA97 in the absence of liver enzymes.

The potential primary metabolites, TBA and propylene glycol did not induce mutations in the bacterial tests.

Mammalian cells in culture

No activity of PGtBE or its potential primary metabolites, TBA and propylene glycol, was observed in experiments with mammalian cells *in vitro*. This lack of activity with PGtBE *in vitro* finds support from studies with PGME that was inactive in a rat primary hepatocyte culture unscheduled DNA synthesis assay (SIDS dossier, 2000), in a Chinese hamster lung V79 cell mutation assay (6-thioguanine resistance) at concentrations up to 5000 µg/ml and in a V79 cell chromosomal aberration assay at concentrations up to 9000 µg/ml (Elias et al., 1996). The last result was confirmed in another chromosomal aberrations study, in which concentrations up to 10000 µg/ml were used, as well as a micronucleus test (dose levels not stated) with Chinese hamster ovary CHO cells (SIDS dossier, 2000). Although Elias et al. (1996) found that PGME induced SCEs in Chinese hamster V79 cells in the absence of S9 enzymes, this statistically significant effect was observed only by an increase from a control level of 10 SCEs/cell to 12 SCEs/cell at the very high concentration of 100 mM. Finally, cellular transformation was not induced in Syrian hamster embryo cells (dose levels not stated) (Elias et al., 1996). Thus, the lack of effect of PGtBE in cultured mammalian cells finds support from a number of studies with a closely related compound.

Mammalian cells *in vivo*

At most, there is weak evidence to suggest that PGtBE might induce micronuclei in mice of one sex, but the evidence is by no means convincing. For the related compound, PGME, the only study *in vivo* for genetic toxicity was where male and female CD-1 mice were administered single intraperitoneal injections of PGME at dose levels up to 6000 mg/kg bw, after which bone marrow cells were examined for micronuclei at 24, 48, 72 h (Elias et al., 1996). The 6000 mg/kg bw dose produced a mortality of 3/8 at 48 h, but there was no increase in the frequency of micronuclei in polychromatic erythrocytes.

The NTP (2003) draft report is suitably cautious in its interpretation of the micronucleus test result and a reading of it does not lead this reviewer to conclude that there is any suggestion that the *in vivo* test result is indicative of a genotoxic mode of action. The draft report quotes a review by Witt et al. (2000) in which it was demonstrated that chemicals that were clearly positive in micronucleus tests conducted to the design that was used in NTP (2003) were highly likely to be carcinogens in rodents. Two important statements can be made regarding this conclusion of Witt et al. (2000). Firstly, it is a correlation of one type of activity with another, not a demonstration of a mode or mechanism of action. Secondly, Witt et al. (2000) determined a clearly positive result in micronucleus tests conducted to this design on the basis of the magnitude of response, or particularly when the response is observed in both sexes of mice. In the case of PGtBE, a statistically significant linear trend of mean MNE frequency across exposure groups was produced only in one sex, so only one of the two possible criteria for a clearly positive response was satisfied (Witt et al., 2000).

Again, the potential primary metabolites, TBA and propylene glycol, have not shown any genotoxic activity in rodent bone marrow assays.

Conclusion

PGtBE induces an increase in mutant numbers in *S. typhimurium* TA97 in the absence of liver enzymes in the incubation mixture; it has no mutagenic effect detectable in this same strain in the presence of liver enzymes or in any other bacterial strain that has been tested in either metabolic activation condition. It has not been unequivocally shown to induce a genotoxic effect *in vivo*, in mouse bone marrow cells. Furthermore, TBA and propylene glycol have not shown any genotoxic potential in a range of assays with bacteria and mammalian cells, either *in vitro* or *in vivo*.

According to the IARC (e.g., 1999) a consistent requirement for establishing a non-genotoxic mode of carcinogenic action is that, "There is a lack of genotoxic activity (agent and/or metabolite) based on an overall evaluation of in-vitro and in-vivo data." This has not been interpreted as meaning that there should be a complete absence of genotoxic activity, even by the IARC, since there are a number of examples of agents (chemicals) where some activity has been demonstrated, but the overall evaluation is that it is unlikely to have contributed to the carcinogenic action. It cannot be concluded, therefore, on the basis of the available data, that a genotoxic mechanism is responsible for

the increase in liver tumours observed with almost identical frequencies in male and female mice.

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ATTACHMENT B

**EXPERT REPORT:
REVIEW OF THE STATISTICAL ANALYSES
OF THE TWO-YEAR RODENT STUDIES
IN THE NTP TECHNICAL REPORT
ON THE TOXICOLOGY AND CARCINOGENESIS STUDIES
OF PROPYLENE GLYCOL MONO-*T*-BUTYL ETHER
(CAS No. 57018-52-7)
IN F344/N RATS AND B6C3F1 MICE
AND A TOXICOLOGY STUDY
OF PROPYLENE GLYCOL MONO-*T*-BUTYL ETHER
IN MALE NBR RATS
(INHALATION STUDIES)
NTP TR 515 PUBLICATION No. 03-449.**

**Ciriaco Valdez-Flores, Ph.D., Larry R. Holden, Ph.D.,
and Robert L. Sielken, Jr., Ph.D.
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**Prepared for
Lyondell Chemical Company**

**Review of the Statistical Analyses of the Two-Year Rodent Studies in the
NTP Technical Report**

**on the
Toxicology and Carcinogenesis Studies of
Propylene Glycol Mono-*t*-Butyl Ether (CAS No. 57018-52-7)
in F344/N Rats and B6C3F₁ Mice
and a Toxicology Study of Propylene Glycol Mono-*t*-Butyl Ether
in Male NBR Rats
(Inhalation Studies)
NTP TR 515 NIH Publication No. 03-4449**

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Introduction

The NTP report is a hazard assessment document and does not include a risk assessment. The purpose of the NTP study is to evaluate the potential toxicological effects of propylene glycol mono-*t*-butyl ether, including carcinogenic activity. It is not the intent of the NTP report to quantitatively characterize either exposures or the risks to humans or animals.

The statistical analyses related to the more critical conclusions drawn in the NTP report have been examined for appropriateness and accuracy. Although the statistical significance of the trends and differences in observed incidences using the poly-3 trend test and reported in terms of p-values could not be reproduced, similar p-values were obtained using equivalent statistical procedures. The p-values reported could not be reproduced exactly because the continuity correction that NTP applied to the poly-3 trend test is not documented in the report or in any of the references cited.

Even though the statistical significance of individual statistical analysis seems appropriate, the overall interpretation of the results has some pitfalls. The report, for example, fails to account for the fact that when there are multiple comparisons not specified a priori, the expected number of false results increases proportionally to the number of comparisons made. In the 2-year inhalation experiments reported by NTP there were more than 100 comparisons for every group of exposed animals. Therefore, the expected number of spurious results (false positives) at the 5% significance level is more than 5 for every group of exposed animals.

The report also fails to present a balanced account of the observed effects. That is, the NTP report discusses and emphasizes the statistically significant increases of adverse effects with dose and excludes from the discussion the statistically significant decreases of adverse effects with dose. Had the NTP properly considered the 'negative' results, which are possibly spurious, their conclusions relating to many 'positive' findings might have been quite different.

NTP draws three separate conclusions from the observed incidences of adverse effects on the 2-year rodent experiments. Our review focuses on the statistical analyses of the data corresponding to the animal experiments that NTP used to conclude that "there was *clear evidence of carcinogenic activity* of propylene glycol mono-*t*-butyl ether in male and female B6C3F1 mice." NTP's three conclusions and our discussion of their statistical analyses are as follows:

1. "There was *clear evidence of carcinogenic activity* of propylene glycol mono-*t*-butyl ether in male and female B6C3F1 mice based on increased incidences of liver neoplasms."

The NTP report concludes that there is clear evidence of carcinogenic activity in male and female mice because of the statistically significant increases in the trend and the incidences of liver neoplasms in mice exposed to 1,200 ppm. The responses leading to the conclusion are listed in Table 1 (Table 20 in the NTP report). All the statistical analyses performed on the response incidences listed in Table 1 are appropriate and correct as far as we can assess. Although the p-values for the poly-3 test listed in the table could not be reproduced exactly, because the continuity correction used by NTP is not documented, the statistical conclusions listed in the table were corroborated using alternative statistical methods. Using Fisher's exact test and the Cochran-Armitage trend test on the adjusted number of animals at risk we obtained the same conclusions and approximately the same p-values as those obtained using the poly-3 test.

The conclusions drawn by the NTP based on the increasing trends of incidences of liver neoplasms are clearly driven by the incidences in male and female mice exposed to the highest dose (1,200 ppm). However, it has been pointed out that the selection of the 1,200 ppm exposure level may be questioned because mice exposed to this concentration showed signs of toxic effects such as ataxia, shallow breathing and lethargy.

No Evidence of Increased Incidence of Liver Effects below 1,200 ppm

If the mice in the highest dose group are excluded from the analyses (Table 2), most of the statistically significant increasing trends disappear and all the incidences that were statistically different at the 1% level from the incidences

in the chamber control group also disappear. Table 1 indicates the incidences of liver effects in mice exposed to propylene glycol mono-*t*-butyl ether for 2 years.

Table 2 shows the incidence of liver effects and the results of the corresponding statistical analyses when the mice exposed to 1,200 ppm are excluded from the analyses. In fact, there are only two effects for male mice exposed to 300 ppm in which the incidences are statistically significantly greater, at the 5% significance level, than the incidences observed in the chamber control mice. Those responses are basophilic focus and hepatocellular adenoma, multiple.

The incidence of basophilic focus in male mice exposed to 1,200 ppm is smaller than the incidence observed in the chamber control male mice and statistically significantly smaller than the incidence in the mice exposed to 300 ppm. Therefore, the statistically significant increase in the incidence of basophilic focus in male mice exposed to 300 ppm should not be interpreted as a dose-related increase.

The second effect (hepatocellular adenoma, multiple) is subsumed in the hepatocellular adenoma that includes multiple. The incidence of this effect (hepatocellular adenoma that includes multiple) for male mice exposed to 300 ppm is not statistically significantly different from the incidence observed in the chamber control male mice.

Excluding basophilic focus and hepatocellular adenoma, multiple, there are no trends that are statistically significant in Table 2. There are, however, a few non-statistically significant trends without the highest dose group. The presence of a few non-statistically significant trends should not be misinterpreted as suggesting a real trend because the multiple trend analyses are not independent.

Spurious Adverse Effects due to Multiple Comparisons

The two instances in which the incidence of effects in male mice exposed to 300 ppm are statistically significantly greater than the incidences in the chamber control male mice shown in Table 2 could be spurious. Furthermore, the slope and the incidences without the mice exposed to 1,200 ppm are statistically significant at the 5% but not at the 1% significance level. The NTP report fails to adjust the significance levels for the multitude of comparisons being made. In male mice alone there were more than 100 effects tested for significant differences in the observed incidences. Tables C.3 to C.5 of the NTP report shows the incidences of all the effects tested for significance in the male mice 2-year experiment.

In a single comparison of incidences there is a 5% chance of erroneously concluding that there is a statistically significant increase, at the 5% level of significance, when in fact there is no effect. That is, at the 5% significance level, one in 20 comparisons are expected to find a spurious effect. The male mice exposed to 300 ppm were compared to the chamber control male mice for more than 100 effects. Thus, finding two spurious statistically significant effects at the 5% significance level in more than 100 comparisons would not be surprising because the expected number of spurious statistically significant effects is more than 5.

Decrease in Frequency of Adverse Effects

Table 20 (summarized version presented here as Table 1) of the NTP report lists only the neoplasms and nonneoplastic lesions of the liver in male and female mice because these are the effects that had statistically significant increase in the incidence of exposed male mice. However, NTP fails to give a balanced account of the effects observed in male mice. An unbiased discussion of the positive and negative findings would give a better perspective on the significance of the increases in observed adverse effects and the relevance of these findings to their conclusions.

Table 3 lists the incidences of alveolar/bronchiolar adenoma, alveolar/bronchiolar carcinoma and the alveolar/bronchiolar adenoma and carcinoma combined in male mice. The incidences of lung tumors decrease with dose. The incidences of alveolar/bronchiolar adenoma and alveolar/bronchiolar adenoma and carcinoma combined in the 300 ppm group are statistically significantly smaller than the corresponding incidences in the chamber control male mice. Again, it would be difficult to conclude that propylene glycol mono-*t*-butyl ether is therapeutic based on these results because it is not surprising to find two additional spurious therapeutic effects in more than 100 comparisons. However, these results show that, just as there can be spurious negative results, the statistically significant increase in the incidences of two liver effects in male mice exposed to 300 ppm could very well be spurious positive results.

Conclusion

The conclusions drawn by the NTP are based solely on some adverse liver effects observed in male and female mice exposed to 1,200 ppm. The mice exposed to 1,200 ppm showed signs of toxic effects and the NTP should consider excluding them when analyzing the incidences of liver neoplasms. If this highest dose group were dropped from the statistical analyses, the only conclusion that could be drawn from the NTP report is that there is no evidence of carcinogenic activity in male or female mice. NTP's conclusion also ignores

some well-known pitfalls of statistical analysis that could have led to spurious statistically significant findings.

Table 1. Incidence of Neoplasms and Nonneoplastic Lesions of the Liver in Mice in the 2-Year Inhalation Study of Propylene Glycol Mono-*t*-butyl Ether listed in Table 20 of the NTP report

Effect	Chamber Control	75 ppm	300 ppm	1,200 ppm
Male Mice				
Number with Liver Examined Microscopically	50	49	50	50
Basophilic Focus	6	11	16*	4
Clear Cell Focus	20	18	16	17
Eosinophilic Focus	9	14	11	29**
Mixed Cell Focus	0	0	0	4
Hepatocyte, Multinucleated	27	23	24	46**
Hepatocellular Adenoma, Multiple	3	7	12*	23**
Hepatocellular Adenoma (Includes Multiple)	18	23	26	36**
Hepatocellular Carcinoma, Multiple	1	1	2	2
Hepatocellular Carcinoma (Includes Multiple)	9	8	13	11
Hepatocellular Adenoma or Carcinoma	25	26	33	41**
Heptoblastoma	0	0	1	5*
Female Mice				
Number with Liver Examined Microscopically	49	50	50	49
Basophilic Focus	3	4	4	2
Clear Cell Focus	4	4	6	5
Eosinophilic Focus	11	10	9	27**
Hepatocellular Adenoma, Multiple	6	0	3	32**
Hepatocellular Adenoma (Includes Multiple)	14	8	10	37**
Hepatocellular Carcinoma, Multiple	0	0	1	2
Hepatocellular Carcinoma (Includes Multiple)	4	8	7	10
Hepatocellular Adenoma or Carcinoma	18	14	16	41**
Heptoblastoma	0	0	0	2

* Incidence is significantly different, at the 5% significance level, from the incidence observed in the chamber control group

** Incidence is significantly different, at the 1% significance level, from the incidence observed in the chamber control group

Table 2. Incidence of Neoplasms and Nonneoplastic Lesions of the Liver in Mice in the 2-Year Inhalation Study of Propylene Glycol Mono-*t*-butyl Ether excluding the group exposed to 1,200 ppm listed in Table 20 of the NTP report

Effect	Chamber Control	75 ppm	300 ppm
Male Mice			
Number with Liver Examined Microscopically	50	49	50
Basophilic Focus	6	11	16*
Clear Cell Focus	20	18	16
Eosinophilic Focus	9	14	11
Mixed Cell Focus	0	0	0
Hepatocyte, Multinucleated	27	23	24
Hepatocellular Adenoma, Multiple	3	7	12*
Hepatocellular Adenoma (Includes Multiple)	18	23	26
Hepatocellular Carcinoma, Multiple	1	1	2
Hepatocellular Carcinoma (Includes Multiple)	9	8	13
Hepatocellular Adenoma or Carcinoma	25	26	33
Heptoblastoma	0	0	1
Female Mice			
Number with Liver Examined Microscopically	49	50	50
Basophilic Focus	3	4	4
Clear Cell Focus	4	4	6
Eosinophilic Focus	11	10	9
Hepatocellular Adenoma, Multiple	6	0	3
Hepatocellular Adenoma (Includes Multiple)	14	8	10
Hepatocellular Carcinoma, Multiple	0	0	1
Hepatocellular Carcinoma (Includes Multiple)	4	8	7
Hepatocellular Adenoma or Carcinoma	18	14	16
Heptoblastoma	0	0	0

* Incidence is significantly different, at the 5% significance level, from the incidence observed in the chamber control group

Table 3. Incidence of Lung Tumors Male Mice in the 2-Year Inhalation Study of Propylene Glycol Mono-*t*-butyl Ether listed in Table C3 of the NTP report

Effect	Chamber Control	75 ppm	300 ppm	1,200 ppm
Male Mice				
Number with Lung Examined Microscopically	50	50	50	50
Lung: Alveolar/bronchiolar Adenoma	13	7	0**	7
Lung: Alveolar/bronchiolar Carcinoma	6	3	1	4
Lung: Alveolar/bronchiolar Adenoma or Carcinoma	17	10	1**	10

** Incidence is significantly different, at the 1% significance level, from the incidence observed in the chamber control group

2. "... there was *equivocal evidence of carcinogenic activity* of propylene glycol mono-*t*-butyl ether in male F344/N rats based on marginally increased incidences of renal tubule and liver neoplasms."

This conclusion is based on the apparent dose-dependent increase in the incidence of renal tubule adenoma and adenoma or carcinoma combined and hepatocellular adenoma in male rats listed in Table 11 and 12 of the NTP report and summarized here in Table 4. However, the trend for the two kidney effects is not statistically significant. The incidences do give a numerically positive trend for male rats exposed to 300 ppm and below. The joint presence of a few non-statistically significant trends, however, should not be over-interpreted as suggesting a real trend. These endpoints and, hence, these multiple trend analyses are not independent.

Overstatement of Increasing Trend of Hepatocellular Adenoma in Male Rats

The analysis of the hepatocellular adenoma responses in male rats deserves more attention because a significant trend was reported. We do not, however, believe the data suggest a chemical effect. The incidences observed in rats exposed to each of the experimental doses were not significantly elevated when compared to the incidence observed in the chamber control male rats. Furthermore, the apparently statistically significantly increasing trend can be easily attributed to the expected random variation between dose groups.

The true incidence of hepatocellular adenomas in this experiment appears to be higher than that the 0.4% level seen in historical controls (1/249, from Table A4c of the NTP report). The probability of observing three or more liver adenomas in male F344/N rats in the chamber controls is equal to 0.001 (1 in 1,000). The group incidences of hepatocellular adenomas observed in the propylene glycol mono-*t*-butyl ether experiment are more elevated, and perhaps more variable, than the incidences observed in the historical controls. Just as there were no hepatocellular adenomas observed in the male rats exposed to 75 ppm there were six observed in the male rats exposed to 1,200 ppm. Both occurrences were not statistically different from the incidence observed in the chamber control male rats. Both of these results are consistent with an experimental background rate closer to the 6% value observed in the chamber control group.

The purported trend in the incidence was statistically significantly increasing at the 5% significance level. However, the significance in the increasing trend could also be considered a more likely result of random differences between groups, not due to the effect of the dose. This apparent

increasing trend is actually due more to the absence of hepatocellular adenomas in the male rats exposed to 75 ppm than to a uniform increase with dose. As stated above, both the lack of hepatocellular adenomas at 75 ppm and the six adenomas observed at 1,200 ppm are consistent with random differences. A certain number of false positive significances are expected. The trend test result is likely to be one of these. Note that if either the lowest exposure group or the highest exposure group were excluded from the trend test, the significance of the "trend" would completely disappear.

It is perhaps important to note that, in addition to simply randomness, the nature of the trend test used can have an effect on apparent significance. With the poly-3 and Cochran-Armitage tests, the statistical significance of a trend is greatly influenced by the placement of the doses. In terms of dose level, the 75 ppm group is numerically closest to the control group than it is to the other dose groups. Thus, the chamber control group and the 75 ppm group are effectively acting like a single point with an incidence of about 3/100. If an alternative version of the Cochran-Armitage test is employed, one using just the ordinal doses instead of actual doses, the trend is no longer statistically significant. The main point here is not that the trend test employed is invalid. Rather, it is merely that such tests are very sensitive to the incidences of dose groups that are at the extremes of the tested dose scale. As a result, one should be very cautious about over-interpreting minor 'trends' as indicative of real dose effects.

Conclusion

The conclusion of equivocal evidence drawn by the NTP is based primarily on the purported increasing slope on hepatocellular adenomas in male rats. However, none of the incidences in the exposed rats are significantly different from the incidence observed in the chamber control male rats. In fact, the incidences at the two lowest dose groups are smaller than the incidence observed in the chamber control rats. The apparently significant trend is more likely a result of random differences in the observed incidences and the unequal spacing between doses. The "significant" trend disappears if the unequal spacing between doses is taken into account by replacing them by ranks. The trend also disappears if the male rats in either the lowest or the highest dose group are excluded from the analysis. These facts make it unlikely that the trend is due to a real chemical effect. Rather, it is more likely due to the peculiar characteristics of the data.

Table 4. Incidence of Kidney Adenomas and Adenomas or Carcinoma in Male Rats in the 2-Year Inhalation Study of Propylene Glycol Mono-*t*-butyl Ether

Effect	Chamber Control	75 ppm	300 ppm	1,200 ppm
Male Rats				
Renal Tubule, Adenoma (includes multiple) Single Sections and Step Sections (Combined)	1 / 50	2 / 50	5 / 49	4 / 50
Renal Tubule, Adenoma or Carcinoma Single Sections and Step Sections (Combined)	1 / 50	2 / 50	5 / 49	5 / 50
Hepatocellular Adenoma ^T	3 / 50	0 / 50	2 / 49	6 / 50

^T Trend is statistically significant at the 5% significance level using the poly-3 trend test

3. "There was *no evidence of carcinogenic activity* of propylene glycol mono-*t*-butyl ether in female F344/N rats exposed to 75, 300, or 1,200 ppm."

Based on this experiment on female F344/N rats, NTP could not have concluded any other way. All the statistical evidence in female mice, if anything, indicates that exposure to propylene glycol mono-*t*-butyl ether results in beneficial effects to female rats.

The conclusion reached by the NTP is based on the fact that there were no statistically significant increases in the incidence of any neoplasms and nonneoplastic lesions in female rats. However, the NTP report fails to discuss in detail the statistically significant negative trends and decreases in the incidences of adenomas or carcinomas of the mammary gland, adenomas of the pituitary gland (unspecified site), and malignant neoplasms of all organs. Furthermore, there were several other decreasing trends of adverse effects that were not discussed in the NTP report. For example, the incidences of mammary gland carcinoma, thyroid adenoma, thyroid adenoma and carcinoma and mononuclear cell leukemia had non-significant decreasing trends. Table 5 lists the adverse effects that had a statistically significant negative trend and some of the responses with a non-significant trend.

The results from the experiment on female F344/N rats should have been used by the NTP to put in perspective the conclusions drawn based on the male and female mice. These apparent beneficial effects, in addition, to the statistically significant decreases in the incidences of some effects on the male mice experiment should have been discussed and factored into the conclusions reported by the NTP.

Table 5. Incidence of Tumors with Negative Trend in Female Rats in the 2-Year Inhalation Study of Propylene Glycol Mono-*t*-butyl Ether listed in Table B3 of the NTP report

Effect	Chamber Control	75 ppm	300 ppm	1,200 ppm
Female Rats				
Mammary Gland: Adenoma or Carcinoma ^T	5 / 50	6 / 50	1 / 50	1 / 50
Pituitary Gland (Unspecified Site): Adenoma ^{TT}	30 / 49	36 / 50	32 / 50	22 / 49*
All Organs: Malignant Neoplasms ^T	33 / 50	27 / 50	32 / 50	23 / 50*
Mammary Gland: Carcinoma	5 / 50	4 / 50	1 / 50	1 / 50
Thyroid Gland (C-Cell): Adenoma	8 / 49	2 / 50	3 / 50	4 / 50
Thyroid Gland (C-Cell): Adenoma or Carcinoma	9 / 49	2 / 50*	3 / 50	4 / 50
All Organs: Mononuclear Cell Leukemia	24 / 50	24 / 50	28 / 50	20 / 50

^T Negative trend is statistically significant at the 5% significance level using the poly-3 trend test

^{TT} Negative trend is statistically significant at the 1% significance level using the poly-3 trend test

* Incidence is significantly different, at the 1% significance level, from the incidence observed in the chamber control group